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Aerosol Stability and Respiratory Infectivity of Lassa Virus

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Running Title: Lassa Virus in Aerosol

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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10 to the 2.67th power

10 to the 4.39th power

ABSTRACT

The aerosol stability and respiratory infectivity of Lassa virus were assessed. Aerosol stability determinations were performed at 24°C and each of three relative humidities (RH) (30, 55, and 80%). A highly significant difference existed between aerosol stability at 30% RH and stability at 55 and 80% RH. Among all RH, 75.3% of the virus in suspension was airborne and infective at 4 min (75% efficiency). Biological half-life values in aerosol of 54.6, 21.5, and 17.5 min were determined for RH of 30, 55, and 80%, respectively. Infectivity of the Josiah strain of Lassa virus for cynomolgus monkeys and outbred guinea pigs was evaluated using dynamic aerosol equipment. All nine cynomolgus monkeys exposed to doses varying from ~~$10^{2.67}$~~ to ~~$10^{4.39}$~~ PFU were infected and died. For outbred guinea pigs, the LD_{50} and ID_{50} values were ~~$10^{3.75}$~~ and ~~$10^{1.18}$~~ PFU, respectively. High titers of infectious virus were recovered from the upper respiratory tract, lungs, and spleen of sequentially killed guinea pigs. Concentrations of virus in the brain and liver were analogous to those in the blood. Transmission, therefore, appeared to occur via the respiratory tract, rather than by direct transport across the cribriform plate to tissues of the central nervous system. Viremia and tissue infection persisted throughout the 30-day study period.

10 to the 1.18 power

10 to the 3.75th power

Lassa virus, an arenavirus, is endemic in western Africa and causes a severe generalized hemorrhagic disease in humans (3, 4, 11). Several outbreaks, with mortality rates of 20 to 40%, have been reported since the initial description of the disease in 1969 (4, 11). Infections also have been induced and described in outbred guinea pigs, and squirrel and rhesus monkeys (7, 13). Recently other animal species were evaluated for susceptibility to Lassa virus infection. Species assessed were inbred guinea pigs and cynomolgus, African green, and capuchin monkeys (P. B. Jahrling et al., manuscript in preparation).

The mode of transmission of Lassa virus has not been elucidated. Epidemiologic evidence, however, suggests that the virus may be disseminated in droplets, with the upper respiratory and gastrointestinal tracts being the portals of entry (3, 11). Development of severe pulmonary lesions in some patients with Lassa fever lends further credence to the theory of airborne transmission. Studies were designed, therefore, to (i) determine the aerosol stability characteristics of Lassa virus, (ii) assess the infectivity of the virus for outbred guinea pigs and cynomolgus monkeys challenged with infectious aerosols, and (iii) partially characterize the pathogenesis of the infections induced.

MATERIALS AND METHODS

Virus. The Josiah strain of Lassa virus, which had been isolated from human serum (14), was used. Inocula were prepared from virus that had been passaged four times in monolayer cultures of Vero cells. Stock virus suspension, containing $10^{6.8}$ PFU/ml, was made in Eagle's minimal essential medium with Earle's salts and nonessential amino acids (EMEM) plus 5% fetal bovine serum (FBS).

Aerosol stability evaluation. Aerosol stability characteristics were defined using methods previously reported (10). For each trial, 1.0 ml of stock virus suspension was disseminated into a 6,200-liter aerosol chamber using an FK-8 atomizer. Aerosols were sampled for 5 min, using standard all-glass impinger (AGI-30) samplers (2), at aerosol ages of 4, 32, and 60 min. Collection fluid in the samplers consisted of 20 ml of EMEM supplemented with 5% FBS, 100 units of penicillin/ml, 100 µg of streptomycin/ml, and 0.1% antifoam (Dow-Corning Antifoam Y-30 Emulsion). Stability of Lassa virus in aerosols was evaluated at 24°C (75°F) and each of three relative humidities (30, 50, and 80% RH).

Animals. Outbred Hartley strain guinea pigs (180-300 g) of either sex were obtained from Buckberg Laboratory Animals, Inc. (Tomkins Cove, NY). Eleven, healthy, clinically normal cynomolgus monkeys, weighing 2.0 to 3.5 kg, also were used. Animals were maintained in appropriate cages and were given water and ration pellets ad libitum throughout the study. All procedures were performed within total containment, gas-tight biological safety cabinets fitted with arm-length rubber gloves.

Respiratory challenge. Animal exposures were performed using dynamic aerosol equipment as described previously (10). When exposing guinea pigs, the Henderson-type aerosol transit tube was modified by incorporation of an animal

exposure box. Cynomolgus monkeys were exposed individually using a head helmet (1) attached to the transit tube. Each animal was exposed for 10 min to an aerosol with 90% of the virus-containing particles of < 4.5 μm. Respiratory minute volumes were estimated using Guyton's formula (5). Total inhaled doses were calculated from the minute volumes and the aerosol concentration of virus delivered.

Virus assay. Plaque assay procedures were used to quantitate virus in suspensions, aerosol samples, and animal tissue specimens. Plaques were enumerated using confluent Vero cell monolayers in 6-well (9.6 cm^2/well) plastic plates (Linbro Scientific Co., Inc., New Haven, CT). Cell cultures were inoculated with 0.2 ml amounts of inoculum, diluted in Hank's balanced salts solution containing 25 mM HEPES buffer, 2% FBS, and antibiotics. Viruses were adsorbed for 1 h at 36°C, then the cultures were overlaid with 3 ml of medium containing Eagle's basal medium with Earle's salts, 4% FBS, 25 mM HEPES buffer, antibiotics, and 0.5% agarose (Sea-Kem; Marine Colloids, Rockland, ME). After the cultures were incubated at 36°C in a 5% CO_2 atmosphere for 3 days, 3 ml of secondary media, comprised of the same medium plus neutral red at a final concentration of 1:10,000, were added. Plaques were counted following incubation for an additional 24 h.

Serology. Assays for humoral antibodies were performed using Jahrling's (6) modification of the indirect immunofluorescence antibody (IFA) procedure originally described by Peters et al. (12). The endpoint was the highest dilution of serum that caused definitive granular fluorescence of the cytoplasm of infected cells.

Pathogenesis. Outbred Hartley guinea pigs (average 192 g) were exposed to aerosols of Lassa virus yielding total inhaled doses of $10^{4.11}$ PFU. Two guinea pigs were killed at each of nine intervals until 30 days after exposure.

Portions of tissue specimens to be tested for presence of virus were homogenized to 30% (w/v) suspensions in the same medium as for aerosol collection. Homogenization was attained using a Model SDT 182N Tissuemizer (Tekmar Co., Cincinnati, OH). The tissue suspensions and blood samples were used as inocula for virus assay.

RESULTS

Aerosol stability. A total of four replicate aerosol trials was evaluated for each of the three RH (30, 55, 80%). Data for these evaluations are summarized in Fig. 1 and Table 1. Among all of the RH, 75.3 ± 9.6% (mean ± SE) of the virus in the stock suspension was airborne and infective at 4 min after dissemination. The differences in aerosol decay rate at 55 and 80% RH were not significant ($P < 0.05$). By contrast, a highly significant difference existed between aerosol stability at 30% RH and stability at either 55 or 80% RH ($P < 0.001$). The mean total decay rates, which include both physical and biological decay, were 2.8, 4.7, and 5.5%/min for RH of 30, 55, and 80%, respectively. Adjustment of these values for physical decay, which has been established previously for our static system to be 1.5%/min (9), yields biological decay rates of 1.3, 3.2, and 4.0%/min for the respective RH (Table 1). These decay rates correspond to Lassa virus biological half-lives in aerosols of 54.6 min at 30% RH, 21.5 min at 55% RH, and 17.5 min at 80% RH.

Respiratory dose-response. When respiratory dose-response titrations were performed in guinea pigs (Table 2), the median lethal dose (LD_{50}) was estimated to be $10^{3.75}$ PFU (95% CL $10^{2.04}$ to $10^{5.46}$). At this challenge dose the mean time to death was approximately 24 days; however, this parameter was not affected appreciably by differences in virus exposure concentrations. By contrast, the median infectious dose (ID_{50}) for guinea pigs was $10^{1.18}$ PFU, with 95% CL of $10^{0.85}$ to $10^{1.52}$. All nine cynomolgus monkeys died that were exposed to concentrations of Lassa virus ranging from $10^{2.67}$ to $10^{4.39}$ PFU; therefore, the LD_{50} for Lassa virus infections in cynomolgus monkeys could not be estimated. The mean time to death was not related to virus exposure dose, and the geometric mean for all nine monkeys was 14.1 days.

Pathogenesis. Guinea pigs that died from Lassa virus infection exhibited

only minimal clinical signs of illness prior to death. Signs were limited to rough hair coats and labored respiration during the terminal 24 to 48 h. Concentrations of virus in selected tissues of guinea pigs following exposure to an inhaled dose of $10^{4.11}$ PFU are shown in Fig. 2 and Table 3. These data suggested that the virus replicated initially in the lungs, then spread via the circulatory system to other organs. High titers of virus were evidenced in the lungs, upper respiratory tract (URT; comprised of mainstem bronchi, trachea, nasal turbinates, and pharynx), and spleen. Concentrations of virus in the brain and liver were similar to those detected in the blood. Viremia and tissue infection were still detectable on day 30 in one of the two guinea pigs examined at that time.

Cynomolgus monkeys given inhaled doses of $10^{2.67}$ to $10^{4.39}$ PFU, similar to guinea pigs, exhibited few overt clinical signs of disease prior to death. Hyperthermia commonly developed 4 to 6 days before death, with rectal temperatures ranging from 39.7 to 40.6°C (103.5 to 105.0°F). Monkeys with lethal infections progressed from a state of inactivity to lethargy during the terminal 72 h. Just prior to death, the monkeys were unable to rise or to transfer food manually to their mouths; approximately 50% developed clonic convulsions a few hours before dying. Histopathologic alterations observed in tissues obtained from two of the monkeys were less severe and extensive than expected for a lethal infection. The principal histopathologic alterations observed were mild, multifocal pneumonia, hepatitis, myocarditis, and choroiditis.

DISCUSSION

Suspensions of Lassa virus in tissue culture medium can be disseminated efficiently as aerosols in particles with a size range capable of remaining airborne and, when inhaled, being deposited in the respiratory tract of animals and humans. Further, the virus is relatively stable after dissemination in aerosol over the range of 30 to 80% RH. Biological decay rates for Lassa virus in aerosol were estimated to range from 1.3 to 4.0%/min. Virus half-lives, therefore, vary from 17.5 min at 80% RH to 54.6 min at 30%. These aerosol stability characteristics exhibited by Lassa virus substantiate the theory of potential hazard of airborne transmission of this virus in laboratory environments and possibly in nature.

Both outbred Hartley guinea pigs and cynomolgus monkeys were susceptible to infection with Lassa virus when it was administered by the airborne route, with an estimated ID_{50} in guinea pigs of 15 PFU. Lethality of the aerosol-induced infection mimicked the mortality results obtained for each species after subcutaneous injection of virus (P. B. Jahrling et al., manuscript in preparation). The mortality rate in outbred guinea pigs never exceeded 50% at any exposure dose up to $10^{3.73}$ PFU, thus precluding establishment of a definitive LD_{50}. Possibly strain 13 guinea pigs would provide a more sensitive model for Lassa virus airborne transmission studies, as they are more sensitive than outbred guinea pigs to infection with Lassa virus (P. B. Jahrling et al., manuscript in preparation) and other arenaviruses (8) when the virus is presented by the subcutaneous route. Additional studies are needed to define the limits of infectivity and lethality of Lassa virus for cynomolgus monkeys challenged via the respiratory route.

Data obtained for virus population dynamics in tissues of outbred guinea pigs infected by the aerosol route were analogous to those reported for strain

13 guinea pigs given virus subcutaneously (P. B. Jahrling et al., manuscript in preparation). Concentrations of Lassa virus in the various tissues, however, were about 10-fold greater in the aerosol exposed outbred guinea pigs, which correlated more closely to the previous findings in rhesus monkeys challenged subcutaneously (7). Apparently increased virus replication must occur in the less susceptible animal before pathologic alterations will develop that lead subsequently to death. The principal sites of viral replication after aerosol exposure appeared to be the lungs and spleen. Evidence was lacking for virus transport from the nasopharynx directly across the foramina of the cribriform plate to the brain. In contrast to the findings with Japanese B encephalitis virus (10), Lassa virus concentrations in the brain were low and probably can be attributed to the existing viremia.

Although the pathogenesis of infection with some microorganisms differs with the portal or mode of entry of the agent, the pathogenesis of Lassa virus appears to be independent of these factors. It is postulated, therefore, that the sequence of virus events following exposure of animals to infectious aerosols is: (i) deposition of infectious virus throughout the respiratory tract followed by initial viral replication in susceptible cells, (ii) development of a viremia that results in hematogenous spread to other organs, and (iii) replication of virus throughout the body with the principal target organ being the spleen. This observation is consistent with reports of Lassa virus infection in guinea pigs (P. B. Jahrling et al., manuscript in preparation) in which the liver did not have a major role in the pathogenesis of disease, as it does in infected humans and primates.

Our data suggest that airborne transmission of Lassa virus in nature is a definite potential. The high concentration of virus ($10^{7.0}$ PFU/g) which developed in the upper respiratory tract, combined with the stability of the

virus in aerosol, provides an outstanding opportunity for distribution of virus in nasal discharges. Thus, the respiratory tract likely may be a principal portal of entry for virus in natural cases of disease.

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Table 1. Effects of relative humidity on percentage recoveries, aerosol decay rates, and aerosol half-lives of Lassa virus

Humidity (%)	Aerosol Recovery (%) Aerosol Age (min) 4	32	60	Biological Decay Rate (%/min)	Biological Half-Life (min)
30	79.6	36.0	16.9	1.3	54.6
55	65.0	11.6	4.5	3.2	21.5
80	83.0	14.4	3.9	4.0	17.5

Table 2. Infectivity, mortality, and time-to-death responses in outbred guinea pigs after aerosol challenge with Lassa virus

Dose (log_{10} PFU)	Dead/total (%)		Seropositive[a]/ survivors	Infected/total (%)		MDTD[b]	(range)
0.70	1/8	(12.5)	0/7	1/8	(12.5)	25.0	
1.68	1/8	(12.5)	6/7	7/8	(87.5)	23.0	
2.86	3/8	(37.5)	5/5	8/8	(100.0)	25.9	(21 - 31)
3.73	4/8	(50.0)	4/4	8/8	(100.0)	24.1	(19 - 31)

[a]Determined by indirect immunofluorescence assay (IFA) using sera obtained 40 days after exposure.

[b]Mean days to death, geometric mean.

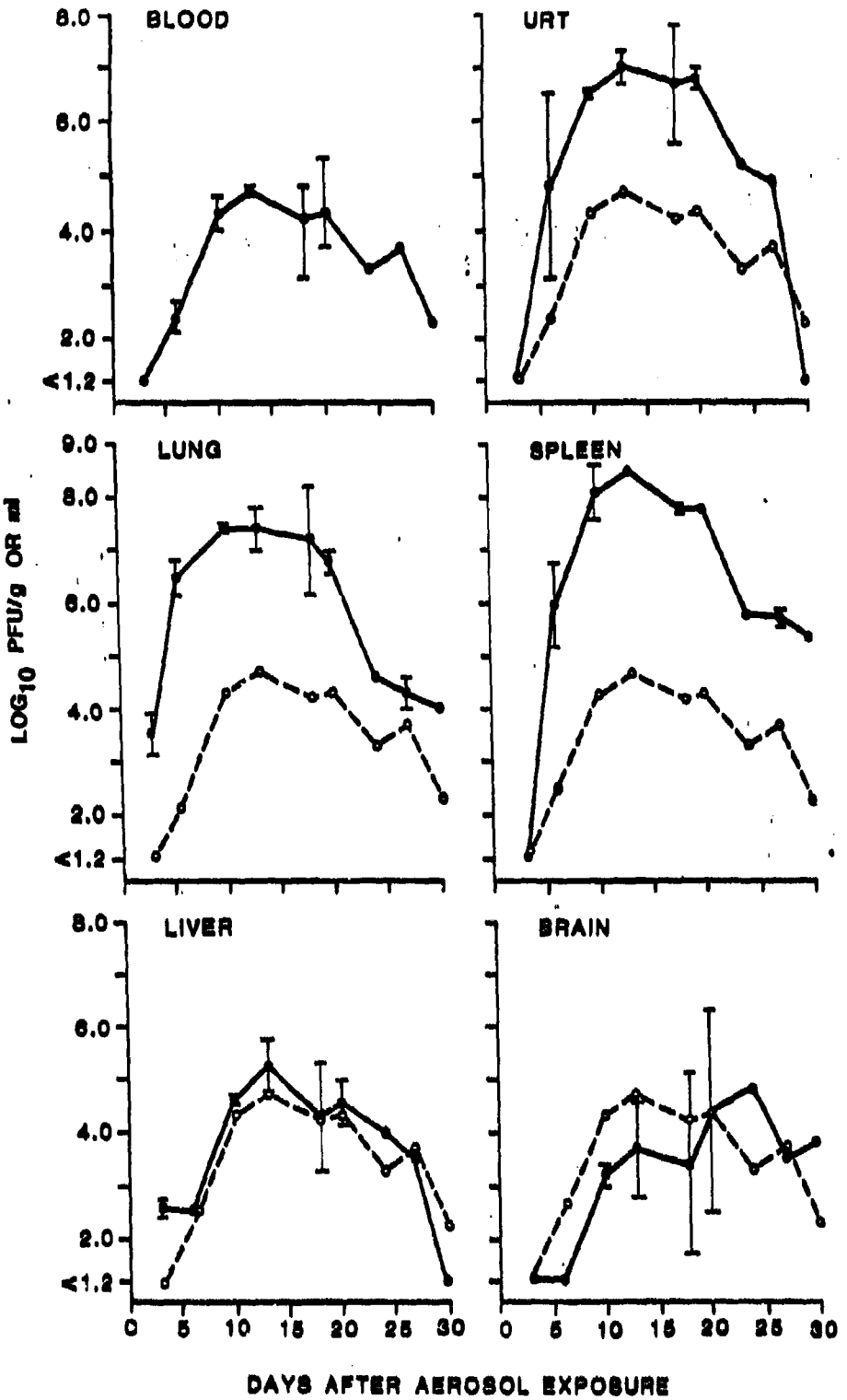
Table 3. Concentrations of infectious Lassa virus in blood and tissues of outbred guinea pigs following aerosol challenge with $10^{4.11}$ PFU

Day After Exposure	Virus Concentration ($\log_{10}$ PFU/g)[a]					
	Blood	URT	Lung	Brain	Liver	Spleen
3	<0.7	<1.2	3.5 (0.4)[c]	<1.2	2.6 (0.2)	<1.8
6	2.4 (0.3)	4.8 (1.7)	6.5 (0.3)	<1.2	2.5[b]	6.0 (0.8)
10	4.3 (0.3)	6.5 (0.0)	7.4 (0.2)	3.2 (0.2)	4.6 (0.1)	8.1 (0.5)
13	4.7 (0.1)	7.0 (0.3)	7.4 (0.4)	3.7 (0.9)	5.3 (0.5)	8.5 (0.0)
18	4.2 (1.1)	6.7 (1.1)	7.2 (1.0)	3.4 (1.7)	4.3 (1.0)	7.8 (0.1)
20	4.3 (1.0)	6.8 (0.2)	6.8 (0.2)	4.4 (1.9)	4.6 (0.4)	7.8 (0.0)
24	3.3[b]	5.2[b]	4.6[b]	4.8[b]	4.0[b]	5.8[b]
27	3.7[b]	4.9 (0.6)	4.3 (0.3)	3.5[b]	3.5[b]	5.8 (0.1)
30	2.3[b]	<1.2[b]	4.0[b]	3.8[b]	<1.2	5.4[b]

[a] Geometric mean of virus concentration in tissue from 2 animals.

[b] One of 2 guinea pigs positive; virus concentration is the amount in the positive animal. On days 24 and 30, all positive tissues were from one animal.

[c] Standard error of mean


BLOOD
URT
LUNG
SPLEEN
LIVER
BRAIN
8.0
6.0
4.0
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10
15
20
25
30
LOG10 PFU/g OR ml
DAYS AFTER AEROSOL EXPOSURE

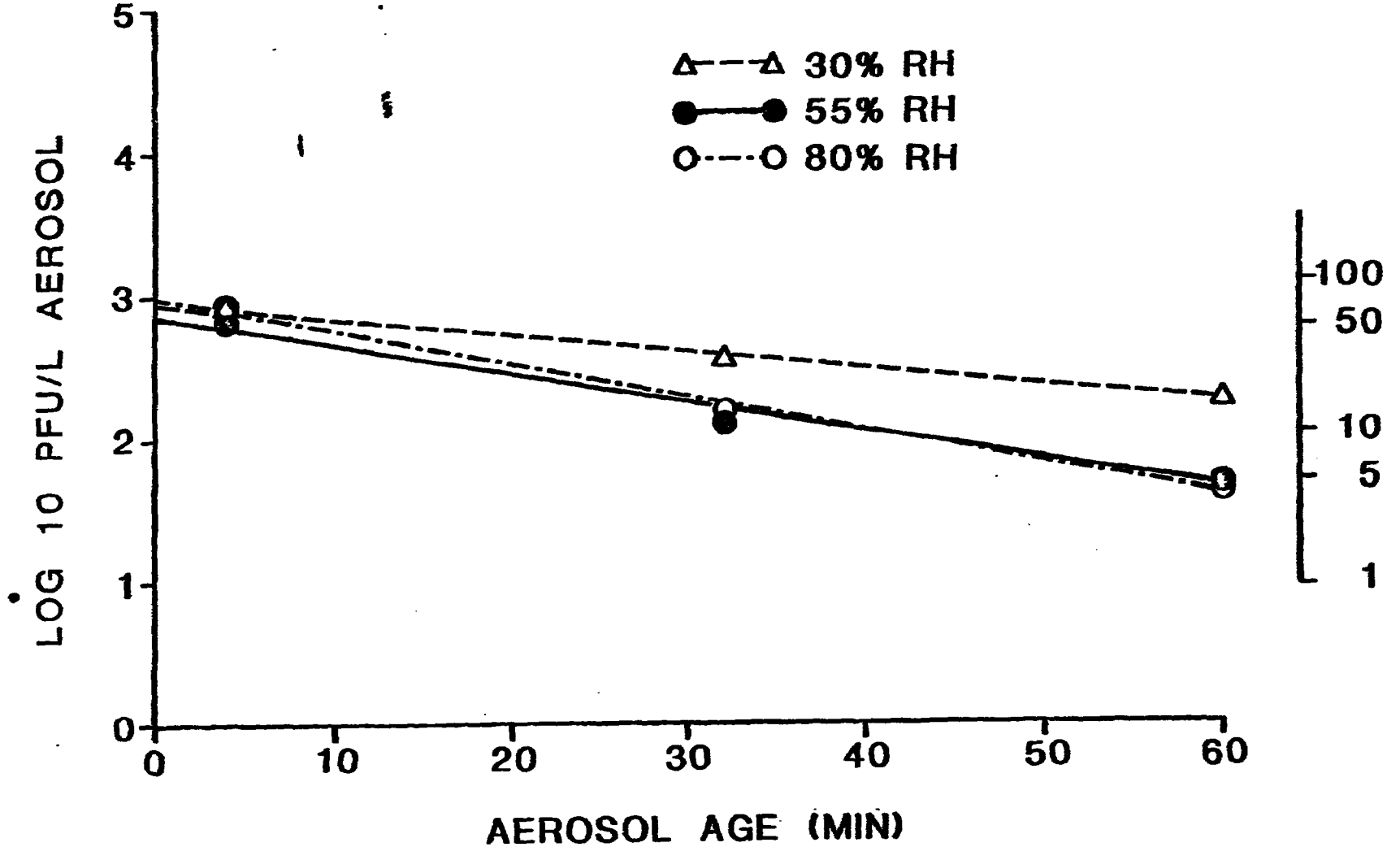
30% RH
55% RH
80% RH
LOG 10 PFU/L AEROSOL
0
1
2
3
4
5
0
10
20
30
40
50
60
AEROSOL AGE (MIN)
% RECOVERY
100
50
10
5
1

FIGURE LEGENDS

Fig. 1. Effect of relative humidity at 24 C (75 F) on aerosol concentration of Lassa virus when 1 ml of virus ($10^{6.8}$ PFU/ml) was disseminated into a 6,200 liter static aerosol chamber and sampled at 4, 32, and 60 min after dissemination.

Fig 2. Concentrations of infectious Lassa virus recovered from blood and tissues of Hartley outbred guinea pigs after aerosol challenge with $10^{4.11}$ PFU. Each point represents the geometric mean (± SEM) for 2 animals. The viremic curve is superimposed on each tissue curve for comparison.

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